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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
26 April 2001 (26.04.2001)

PCT

(10) International Publication Number
WO 01/28588 A1

(51) International Patent Classification[7]: A61K 45/06, A61P 9/10, A61K 31/232, 31/23

(21) International Application Number: PCT/CA00/01242

(22) International Filing Date: 16 October 2000 (16.10.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
2,286,442 15 October 1999 (15.10.1999) CA

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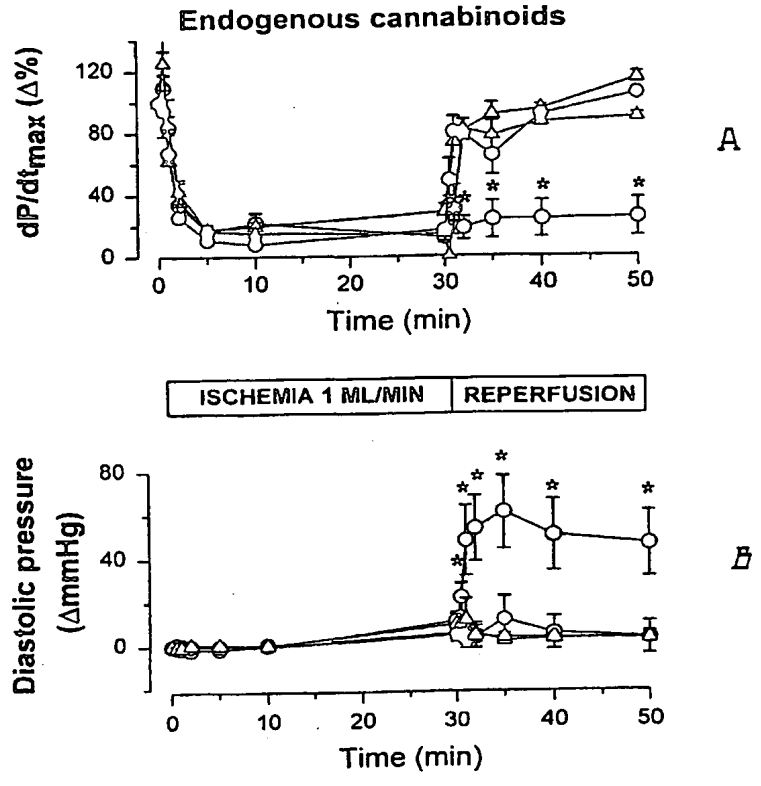
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,

[Continued on next page]

(54) Title: CANNABINOIDS AS VASODILATORS AND CARDIOPROTECTORS AGAINST ISCHEMIA



(57) Abstract: The potent anti-ischaemic effects of cannabis-like drugs in the brain and the presence of CB_1 and CB_2-type cannabinoid receptors in heart indicate that endogenous cannabinoids may contribute to protect the heart against the deleterious effects of ischemia and reperfusion. 2-arachidonylglycerol and palmitoyl-ethanolamide abolished the deleterious effects produced by ischemia and reperfusion by interacting with cannabinoid receptors located in heart the cannabinoid effects were antagonized cannabinoids by receptor antagonists. These results indicate that peripheral CB_2 receptors participate in the intrinsic defense of the heart against ischaemic insult and their endogenously and locally produced agonists such as 2-arachidonyl-glycerol and palmitoylethanolamide may mediate this protective effect. Ischemic preconditioning affords protection to the endothelial function in resistance coronary arteries of the rat partially by activation of CB_1 and CB_2 receptors. Exogenous cannabinoid perfusion protected the endothelium via CB_1 and/or CB_2 receptors.

WO 01/28588 A1

IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

— *With international search report.*

— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

TITLE OF THE INVENTION

Cannabinoids as vasodilators and cardioprotectors against ischemia.

HISTORY OF THE INVENTION

Cardiovascular disease is a serious health problem and a prime cause of death in most developed countries and the cost associated with this disease or its complications is high. Currently, therapy of cardiovascular disease is not totally efficient against mortality and morbidity associated to this disease. Moreover, the current therapy is completely inefficient to prevent the initiation and the development of the myocardial infarction.

Delta9-Tetrahydrocannabinol, the psychoactive marijuana-derived cannabinoid, binds to the CB_1 and the CB_2 receptors. Arachidonyl ethanolamide (anadamide), palmitoyl ethanolamide (PEA) and 2-arachidonyl glycerol (2-AG) are naturally-occurring brain and heart constituents that act as CB_1 and CB_2 agonists and exhibit pharmacological activity comparable to cannabinoids. Compounds which stimulate the CB_1 receptor have been shown to induce analgesia. Cannabinoids have also been shown to suppress the immune system and may act as antioxydant agents. Thus, compounds which stimulate the CB_1 and CB_2 receptors, directly or indirectly, are potentially useful during myocardial infarction or during cardiac transplant, preventing the development of infarction, avoiding tissue rejection, and controlling associated pain. Compounds which stimulate only the CB_2 receptor may have the same protective effect without analgesia or drowsiness side effects. These latter compounds will be potentially useful in the prevention of cardiovascular diseases.

The present invention relates to the use of naturally occurring cannabinoid compounds, their active metabolites and related synthetic compounds, and any agonist of their receptors, in the treatment or prophylaxis, or in the making of a medication for the treatment or prophylaxis of cardiovascular diseases in humans and

animals. Other aspects of the compounds covered by this invention are protective compounds having an effect through the increase in the synthesis of cannabinoids and/or the decrease of their degradation and/or the decrease of their uptake that would make them disappear from their site of action and/or the activation of their specific receptors. All the above compounds are grouped under the terms "cannabinoids like" compounds.

The present inventors have found that the endogenous protection of myocardium or endothelium afforded by endogenously produced cannabinoids can be blocked by selective antagonists of cannabinoid receptors. Furthermore, when perfused exogenously, these compounds protected the myocardium and the endothelium against ischemia and reperfusion. This exogenous protection was also blocked by selective antagonists of cannabinoid receptors.

Thus, cannabinoids or their derivatives will be potentially helpful in the treatment or the prophylaxis of cardiovascular diseases. For example, they will be useful to prevent the development of myocardial infarction, either during ambulance transportation of a patient suffering of heart attack, or in a given population of patients at risk. Currently, only few drugs, if any, have the capacity of relieving pain and protecting myocardium and endothelium against ischemic insult.

Therefore, in accordance with the present invention is provided a method for protecting endothelial cells or cardiomyocytes against damages caused by ischemia, which comprises the step of contacting the cells or myocytes with a protective amount of a cannabinoid-like compounds e.g. any compound contributing to the increase in the extracellular concentration of cannabinoids or mimicking the same.

The compound may be capable of increasing the synthesis of endogenous cannabinoids or of decreasing the degradation

or the disappearance thereof, or is a cannabinoid active metabolite, a cannabinoid analog or a cannabinoid agonist.

The compound is more specifically PEA, 2-AG, or L 768242, L 759633, L 759656, JWH-015, JWH-05 or JWH-057, or a mixture thereof. In a most specific embodiment, endogenous cannabinoids habe been exogenously administered.

Any new composition useful in the practice of the above method is within the scope of the invention. Further, the use of same compound in the making of a medication for the same purpose as the one fulfilled with the above method is also within the scope of the invention.

This invention will be described hereinbelow, referring to specific embodied examples and appended figures, which purpose is to illustrate the invention rather than to limit its scope.

BRIEF DESCRIPTION OF THE DRAWINGS

Having thus generally described the invention, reference will now be made to the accompanying drawings, showing by way of illustration a preferred embodiment thereof, and in which:

Figure 1

Endogenous cannabinoids protect myocardial function against the deleterious effect of ischemia and reperfusion. **A.** Effects of 1μM SR 141716A, 3 μM AM630 (CB_1 receptor selective antagonists) and 1μM SR 144528 (CB_2 receptor selective antagonist) on dP/dt_{max}, an index of myocardial contractility during the 30 min (flow 1 ml min^{-1}) ischemia and 20 min reperfusion. **B.** Effects of 1μM SR 141716A, 3 μM AM630, and 1μM SR 144528 on diastolic pressure, an index of contracture under the same experimental conditions. Closed circle, open circle, closed triangle, open triangle represent respectively ischemia without cannabinoid antagonist (n=8), treated with SR 144528 (n=5), treated with SR 141716A (n=6), and treated AM 630 (n=6). Asterisk indicates $P<0.05$ vs ischemia without CB_2 or CB_1 antagonists.

Figure 2

PEA affords cardioprotection by activating peripheral CB_2 cannabinoid receptors. **A.** Effects of 300nM PEA in the absence or presence of the CB_1 receptor selective antagonist SR 141716A 1μM or the CB_2 receptor selective antagonist SR 144528 1μM on dP/dt_{max}, an index of myocardial contractility during the 120 min (flow 0.5 ml min^{-1}) ischemia and 20 min reperfusion. **B.** Effects of 300nM PEA in the absence or presence of the CB_1 receptor selective antagonist SR 141716A 1μM or the CB_2 receptor selective antagonist SR 144528 1μM on diastolic pressure, an index of contracture during the same experimental conditions. Closed circle, open circle, closed triangle, open triangle represent respectively ischemia and reperfusion without PEA (n=6), with PEA (n=5), with PEA pretreated with SR 141716A (n=5), and with PEA pretreated with SR 144528 (n=5). Asterisk indicates $P<0.05$ vs ischemia without PEA. **C, D** represent respectively the activity of LDH and CK (indexes of cell sufferance) measured in the coronary effluent at the end of the 20 min reperfusion period. Open, horizontally hatched, hatched, cross-hatched, and vertically hatched columns represent hearts without ischemia (n=5), with ischemia (n=5), with ischemia + PEA (n=5), with ischemia + PEA pretreated with SR141716A (n=5), and with ischemia + PEA pretreated with SR144528 (n=5). Asterisk indicates $P<0.05$ vs without ischemia group.

Figure 3

2-AG affords cardioprotection by activating peripheral CB_2 cannabinoid receptors. **A.** Effects of 300nM 2-AG in the absence or presence of the CB_1 receptor selective antagonist SR 141716A 1μM or the CB_2 receptor selective antagonist SR 144528 1μM on dP/dt_{max}, an index of myocardial contractility during the 120 min (flow 0.5 ml min^{-1}) ischemia and 20 min reperfusion. **B.** Effects of 300nM 2-AG in the absence or presence of the CB_1 receptor selective antagonist SR 141716A 1μM or the CB_2 receptor selective antagonist SR 144528 1μM on diastolic pressure, an index of contracture under the same experimental conditions. Closed circle, open circle, closed triangle,

open triangle represent respectively ischemia and reperfusion without 2-AG (n=6), with 2-AG (n=6), with 2-AG pretreated with SR 141716A (n=5), and with 2-AG pretreated with SR 144528 (n=5). Asterisk indicates $P<0.05$ vs ischemia without 2-AG. **C, D** represent respectively the activity of LDH and CK (indexes of cell sufferance) measured in the coronary effluent at the end of the 20 min reperfusion period. Open, horizontally hatched, hatched, cross-hatched, and vertically hatched columns represent hearts without ischemia (n=5), with ischemia (n=5), with ischemia + 2-AG (n=5), with ischemia + 2-AG pretreated with SR141716A (n=5), and with ischemia + 2-AG pretreated with SR144528 (n=5). Asterisk indicates $P<0.05$ vs without ischemia group.

Figure 4

Anadamide cannot afford cardioprotection. **A.** Effects of 3μM anandamide on dP/dt_{max}, an index of myocardial contractility during the 120 min (flow 0.5 ml min^{-1}) ischemia and 20 min reperfusion. **B.** Effects of anandamide on diastolic pressure, an index of contracture during the same experimental conditions. Closed and open circles represent respectively ischemia and reperfusion without anadamide (n=6) and with anandamide (n=4). **C, D** represent respectively the activity of LDH and CK (indexes of cell sufferance) measured in the coronary effluent at the end of the 20 min reperfusion period. Open, horizontally hatched, and hatched columns represent hearts without ischemia (n=5), with ischemia (n=5), and with ischemia + anandamide (n=5).

Figure 5

Diagrams showing the different experimental protocols. Each experiment started with a 20-min stabilization period. Hearts in the ischemia protocol (no. 2 & 5) underwent 30 min of low-flow (1 ml min^{-1}) ischemia and 20 min of reperfusion, after an additional 30-min stabilization period. Hearts in the ischemic preconditioning + ischemia protocol (no. 3) were submitted to a preconditioning 5 min zero-flow ischemia and 10 min reperfusion, before the 30-min low-flow

ischemia. Perfusion of drugs (SR141716A 1μM (no.1-5), AM630 3μM (no.1-3), SR144528 1μM (no.1-5) or their respective vehicles) was started after the 20-min stabilization period, lasted throughout the 30-min low-flow ischemia or a corresponding period, and was stopped upon reperfusion. Perfusion of 2-arachidonoylglycerol (2-AG,300nM), palmitoylethanolamide (PEA, 300nM), anandamide (AEA, 100nM-1μM) or their respective vehicle (no.4 & 5) was started 15 min before and lasted throughout the 30-min low-flow ischemia or a corresponding period. For all protocols, endothelial and smooth muscle function was evaluated after the 20-min reperfusion period. Coronary arteries were precontracted by a continuous infusion of 0.1μM U-46619. After 15 min, infusion of 5-HT (10μM) was started for 10 min. A wash-out period of 10 min was allowed between 5-HT and SNP (3μM, 10 min) infusions. The bottom axis represents the time in min.

Figure 6

Change in coronary resistance (Δ%) induced by 10μM serotonin (5-HT, panels A, C, and E) and 3μM sodium nitroprusside (SNP, panels B, D, and F) in untreated hearts (panels A and B), in 1μM SR141716A (panels C and D), and in 1μM AM630 pretreated hearts (panels E and F). Open, cross-hatched, and hatched columns represent sham, ischemic, and ischemic preconditioning protocols, with 8, 8, and 9 untreated, 10, 8, 6 SR141716A and 8, 8, and 6 AM 630 pretreated hearts, respectively. ¤ $p<0.05$, compared with sham and ischemic preconditioning. *$p<0.05$, compared with sham (one way ANOVA with Scheffé post-hoc test).

Figure 7

Change in coronary resistance (Δ%) induced by 10μM serotonin (5-HT, panels A and C) and 3μM sodium nitroprusside (SNP, panels B and D) in untreated hearts (panels A and B), and in 1μM SR144528 pretreated hearts (panels C and D). Open, cross-hatched, and hatched columns represent sham, ischemia, and ischemic preconditioning protocols, with 8, 8, and 9 untreated, and 9, 8, and 6

SR144528 pretreated hearts, respectively. ¤ $p<0.05$, compared with sham and ischemic preconditioning. * $p<0.05$, compared with sham. (one way ANOVA with Scheffé post-hoc test).

Figure 8

Change in coronary resistance (Δ%) induced by 10μM serotonin (5-HT, panels A, C, and E) and 3μM sodium nitroprusside (SNP, panels B, D, and F) in anandamide (AEA, panels A and B), in palmitoylethanolamide (PEA, panels C and D), and in 2-arachidonoylglycerol (2-AG) pretreated hearts (panels E and F). Open, hatched, cross hatched horizontally hatched, and vertically hatched columns represent respectively in panels A and B sham, ischemia w/o AEA, ischemia with AEA 100nM, ischemia with AEA 300nM, and ischemia with AEA 1μM. In panels C and D, they represent respectively sham, ischemia w/o PEA 300nM, ischemia with PEA 300nM + SR141716A 1μM, and ischemia with PEA 300nM + SR144528. Finally, in panels E and F, they represent sham, ischemia w/o PEA 300nM, ischemia with PEA 300nM + SR141716A 1μM, and ischemia with PEA 300nM + SR144528 respectively. *$p<0.05$, compared with sham (one way ANOVA).

EXAMPLE 1

Cardioprotection is induced by cannabinoids

Cardiovascular disease is a serious health problem and a prime cause of death in most developed countries and the cost associated with this disease or its complications is high [1]. Currently, therapy of cardiovascular disease is not totally efficient against the mortality and morbidity associated with this disease.

Numerous studies have demonstrated that exogenous perfusions of endogenously produced agents such as adenosine [2], kinins [3], prostaglandins [4], or opioids [5] afford protection against the deleterious effects of ischemia and reperfusion on myocardial function.

Delta9-Tetrahydrocannabinol, the psychoactive marijuana-derived cannabinoid, binds to CB_1 and CB_2 receptors [6]. The

existence of these receptors implies the presence of endogenous ligands. Arachidonylethanolamide (anandamide)[7], palmitoylethanolamide (PEA) [8], and *sn*-2 arachidonylglycerol (2-AG) [9,10] are naturally-occurring constituents in several organs that act as CB_1 and/or CB_2 agonists and exhibit pharmacological activity comparable to cannabinoids. Compounds which stimulate the CB_1 and/or the CB_2 receptors have been shown to induce analgesia [11]. Cannabinoids have also been shown to suppress the immune system [8,12] and may act as antioxydant agents [13]. Furthermore, these compounds may have some cardiovascular properties[14,15] and the presence of $_m$ARN coding for their specific receptors have been detected in heart [16]. All these properties made cannabinoids good candidates for cardioprotection.

To determine whether endogenous cannabinoids have an analogous function to that of adenosine, kinins, opioids, and prostaglandins on cardioprotection, we used the Langendorff isolated rat heart model submitted to a 30 min low-flow ischemia (flow 1ml min^{-1}) followed by a 20 min reperfusion pre-treated with CB_1 (3µM AM630 or 1µM SR141176A) or CB_2 receptor antagonists. The dP/dt_{max} has been used as an index of myocardial contractility and the diastolic pressure as an index of contracture. Taken together, these end points served to evaluate the recovery of myocardial function.

Methods

Drugs

SR 144528 and SR 141716a were a kind gift from Sanofi Recherche, Montpellier, France; anandamide, and 2-AG were from Sigma-Aldrich (Mississauga, Ont, Canada); PEA and AM 630 were provided by Tocris Cookson (Ballwin, MO, USA). AM 630 (9.9mM), was prepared in 1ml 100 % dimethylsulphoxide (DMSO) and 1 ml propylene glycol. SR 144528 and SR 141716A (10mM) were prepared in 1ml 100 % DMSO. All these stock solutions were diluted in bidistilled water to obtain the desired final concentrations. Anandamide (1mM) was diluted in 1ml propylene glycol and 9 ml of Krebs-Henseleit buffer. 2-

arachidonoylglycerol (13.2 mM) and palmitoylethanolamide (16.7mM) were dissolved in anhydrous ethanol. All these stock solutions were diluted in Krebs-Henseleit buffer to obtain the desired final concentrations.

Ischemia and reperfusion procedure

Male Sprague-Dawley rats (300-350 g) were narcotized with CO_2 until a complete loss of consciousness and rapidly decapitated. Hearts were rapidly excised and immersed in ice-cold heparinised Krebs-Henseleit buffer (10 IU ml^{-1}). They were immediately mounted on the experimental Langendorff setup and perfused at constant flow by means of a digital roller pump. The flow rate was adjusted during the stabilization period to obtain a coronary perfusion pressure of approximately 75 mmHg and was held constant, with the exception of the ischemic periods during which flow was either reduced to 1 ml min^{-1} (30 min ischaemic period) or 0.5 ml min^{-1}(120 min ischaemic period). Flow rate was measured during all the experiment with an in-line ultrasonic flow probe and meter (Transonic Systems Inc., model T106). The normal perfusion solution consisted of a modified Krebs-Henseleit buffer containing (in mM): NaCl 118, KCl 4, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1, $NaHCO_3$ 24, D-glucose 5, pyruvate 2. The perfusate was gassed with 95% O_2- 5% CO_2 (pH 7.4) and kept at a constant temperature of 37 ^{0}C. All drugs were administered through a Y connector in the aortic cannula with syringe pumps (Harvard Apparatus, model 11) at one hundredth of the coronary flow rate. Adequate mixing of the drugs was ensured by the turbulent flow created in the reverse drop shaped aortic cannula. All concentrations mentioned in the text and figures refer to the final concentration after mixing. Isovolumetric left ventricular pressure and its first derivative (dP/dt) was measured by a fluid filled latex balloon inserted into the left ventricle and connected to a pressure transducer. The volume of the balloon was adjusted to obtain a diastolic pressure between 5 and 10 mmHg. Data were recorded on a polygraph system (Grass Model 79 polygraph).

The hearts in all groups were subjected to a 20-min stabilization period. Drugs or vehicle infusion was then started, followed by an additional 15-min perfusion period. The ischaemic groups were subjected to a 15-min sham period, followed by either 30 min of partial ischemia (flow rate 1 ml min^{-1}) or 120 min of partial ischemia (flow rate 0.5 ml min^{-1} prior to a 20-min reperfusion period.

Ischaemic hearts were treated with either 1μM SR144528, 1μM SR141716A, or their respective vehicles starting after the 20-min stabilization period, and lasting throughout the 30-min ischaemic period. Drug infusion was stopped upon reperfusion.

In additional experimental series, the effect of an exogenous PEA, 2-AG, anandamide, or vehicle perfusion was studied. In these groups, hearts were pretreated with either 1μM SR144528, 1μM SR141716A or vehicles starting after the 20-min stabilization period, in order to expose the hearts to 15-min antagonist perfusion before the exposition to exogenous cannabinoid agonists. The antagonist perfusion lasted throughout the 120-min ischaemic period, and was stopped upon reperfusion. Exogenous cannabinoids were perfused 15 min before the 120-min low-flow ischemia and was stopped upon reperfusion.

CK and LDH determination in coronary effluent

Coronary effluent samples were collected at the end of reperfusion period. All the samples were then stored at –80°C until analysis. The activity of CK and LDH in coronary effluent samples were evaluated by Sigma diagnostic procedures (procedure 520 for CPK and procedure 228-UV for LDH).

Data analysis

Results are expressed as means± s.e.m.. Th significance of differences between groups was evaluated using analysis of variance with Scheffé post-hoc test.

Results

In the groups non-exposed to cannabinoid receptor antagonists or exposed to CB_1 antagonists (AM 630 or SR141716A), the dP/dt_{max} decreased during ischemia and rapidly recovered upon reperfusion (fig 1A). Diastolic pressure remained unchanged during all the experiment indicating an absence of contracture (fig. 1B). Thus, in these groups, myocardial function recovered completely from the ischaemic and reperfusion insult.

In the group pre-treated with the CB_2 antagonist (1μM SR144528), the hearts did not resume spontaneous beating upon reperfusion (no recovery of dP/dt_{max}) (fig 1A). Also, the reperfusion was accompanied by a huge increase in diastolic pressure, indicating the presence of a severe contracture (fig. 1B). These results indicate that endogenous cannabinoids play a major role in the protection of the myocardium against ischemia and reperfusion insult. Moreover, this protective effect is mediated via the activation of CB_2 receptors.

To determine whether exogenous cannabinoids can preserve the myocardium against the deleterious effect of a more severe ischemia, we used rat hearts submitted to a 120 min low-flow ischemia (flow 0.5 ml min^{-1}) followed by a 20 min reperfusion. Again, dP/dt_{max} has been used as an index of myocardial contractility and diastolic pressure as an index of contracture. Also we measured the activity of creatine phosphokinase (CK) and the activity of lactate dehydrogenase (LDH) present in the coronary effluent at the end of the 20-min reperfusion period. An increased outflow of these intracellular enzymes in the coronary effluent indicates the severity of cell damage, for our particular case, the importance of the myocardial infarct. Under these more severe ischaemic conditions, we observed, in the group

exposed only to ischemia an reperfusion, no recovery of dP/dt_{max} (fig. 2A), an increase in diastolic pressure (fig 2B), and of activities of LDH (fig. 2C) and CK (fig. 2D) in the coronary effluent, indicating severe deleterious effects of ischemia and reperfusion.

The first endogenous cannabinoid evaluated was PEA, an acylethanolamide found in neural and non-neural tissues, which inhibits mast-cell activation and reduces inflammatory responses [12,17] by a mechanism that may involve binding to CB_2 receptors [8]. We found that 300 nM PEA, when perfused 15 min before ischemia and lasting throughout the ischaemic period, is able to protect hearts from ischemia. Perfusion of PEA improved dP/dt_{max} recovery (fig. 2A), decreased myocardial contracture (prevented the increase in diastolic pressure during reperfusion)(fig 2B), and avoided the increase in LDH (fig. 2C) and CK (fig. 2D) activities. Under our experimental conditions, the protective effect afforded by the perfusion PEA was reversed by 1μM SR144528 (CB_2 antagonist), whereas the CB_1 antagonist SR141716A was ineffective (fig 2A, B, C, D). Taken all together, these results indicate that PEA exerts cardioprotective effects that are mediated by peripheral CB_2 receptors. For the moment, the cellular location of such receptors is unknown.

The second endogenous cannabinoid evaluated was 2-AG. We found that 300 nM 2-AG when perfused 15 min before ischemia and lasting throughout the ischaemic period is able to protect hearts from ischemia. Perfusion of 2-AG increased dP/dt_{max} recovery (fig. 3A), decreased myocardial contracture (prevented the improved in diastolic pressure during reperfusion)(fig 3B), and avoided the increase in LDH (fig. 3C) and CK (fig. 3D) activities. SR144528 but not SR141716A reversed the protective effect produced by 2-AG (fig 3A, B, C, D). As with PEA, these results indicate that 2-AG exerts its cardioprotective effects via the stimulation of peripheral CB_2 receptors.

Exogenous perfusion of anandamide did not produce any cardioprotective effects (fig 4A, B, C, D). This lack of effect may be

explained by the short life span on this compound, which undergoes rapid inactivation in tissues [18,19].

Our results show that the activation of local CB_2 cannabinoid receptors may protect rat hearts from the deleterious effects of ischemia and reperfusion. These findings suggest the possibility that endogenously produced cannabinoid agonists such as palmytolethanolamide and *sn*-2 arachidonylglycerol may afford natural protection to the heart against ischemia and reperfusion.

Furthermore, these results indicate that selective CB_2 receptor agonists perfused exogenously may reduced the damages produced by ischemia and reperfusion.

Thus, cannabinoids or their derivatives will be potentially helpful in the treatment or the prophylaxis of cardiovascular diseases. For example they will be useful to prevent the development of myocardial infarction, either during ambulance transportation of a patient suffering of heart attack, or in a given population of patients at risk. Currently, only few drugs if any have the capacity of relieving pain and protecting myocardium against ischaemic insult.

EXAMPLE 2

Cannabinoids preserve endothelium-dependent vasodilation

In recent years a great deal of interest has focused on the phenomenon of ischemic preconditioning and the mechanisms by which its potent cardioprotective effect occurs. This phenomenon described for the first time by Murry et al [20] limits infarct size [20; 21], reduces the risk of ischemia-reperfusion arrhythmias [22; 23], improves recovery of ventricular function [21], reduces catabolite accumulation, and slows ischemic metabolism [24; 25].

In addition, it has been shown that the beneficial effect of IPC is not limited to the cardiomyocytes, but can be observed in endothelial cells in various experimental models including dog resistance coronary arteries *in vivo* [26], and coronary circulation of the rat *in vitro* [27; 28]. Adenosine [28-30], des-Arg^{28}-bradykinin [31], prostaglandin E_2

(unpublished data), opioids [32], ATP-sensitive potassium channels (K_{ATP} channels) [28; 33; 34], and protein kinase C (PKC) activation [35; 36] have all been implicated as mechanisms of the protection afforded by IPC.

Delta^9-Tetrahydrocannabinol, the psychoactive marijuana-derived cannabinoid, binds to CB_1 and CB_2 receptors [37]. The existence of these receptors implies the presence of endogenous ligands. Arachidonylethanolamide (AEA) [7], palmitoylethanolamide (PEA) [8], and *sn*-2 arachidonoylglycerol (2-AG) [9; 10] are naturally-occuring constituents of the membrane of several organs that act as CB_1 and/or CB_2 agonists and exhibit pharmacological activity comparable to cannabinoids. Cannabinoids have also been shown to suppress the immune system [8; 12] and may act as antioxydant agents [13]. Furthermore, these compounds may have some cardiovascular properties[14; 15] and the $_m$ARN coding for their specific receptors has been detected in heart [16]. All these properties made cannabinoids good candidates for cardioprotection.

However, for the moment, little is known about the role played by endogenous cannabinoids in the endothelial protective effect of IPC. Therefore, the first aim of the present study was to evaluate whether IPC affords protection against ischemic injury to the endothelium of coronary vessels in isolated rat hearts via cannabinoid receptor activation. The second aim was to verify whether exogenous cannnabinoid perfusion could mimic the beneficial effects of IPC against ischemic injury in these hearts.

Methods

Preparation of hearts

The investigation was performed in accordance with the guidelines from the Canadian Council on Animal Care. The detail methodology has been described earlier [9],male Sprague-Dawley rats (300-350 g) were narcotized with CO_2 until a complete loss of consciousness and rapidly decapitated. Hearts were rapidly excised and perfused at constant flow by means of a digital roller pump. A 20-

ml compliance chamber along the perfusion line ensur d a continuous flow (Langendorff perfusion). The flow rate was adjusted during th stabilization period to obtain a coronary perfusion pressure of approximately 75 mmHg and was held constant, with the exception of the ischemic periods during which flow was either stopped (zero-flow ischemia) or reduced to 1 ml min^{-1} (low-flow ischemia). A second adjustment of the flow rate was made at the end of the long reperfusion period, before the perfusion of U-46619, to correct any deviation of the coronary perfusion pressure from 75 mmHg, and was held constant thereafter. Perfusion pressure and flow rate were monitored to calculate coronary resistance. The perfusion solution (a modified Krebs-Henseleit buffer) contained (in mM): NaCl 118, KCl 4, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1, $NaHCO_3$ 24, D-glucose 5, pyruvate 2, gassed with 95% O_2-5% CO_2 (pH 7.4), 37 ^{o}C. All drugs were administered through a Y connector in the aortic cannula with syringe pumps.

Experimental protocols

Ischemic preconditioning protocol

The animals were assigned to one of three different experimental protocols (fig 5). The hearts in all groups were subjected to a 20-min stabilization period. Drugs or vehicle infusion was then started, followed by an additional 15-min perfusion period. The ischemic groups (protocol 2, fig 5) were subjected to a 15-min sham period, followed by 30 min of partial ischemia (flow rate 1 ml/min) prior to a 20-min reperfusion period. In the preconditioned groups (protocol 3, fig 5), the hearts were exposed to 5 min global ischemia (zero flow) plus 10 min of reperfusion before the 30-min ischemia and 20-min reperfusion periods. The sham groups (protocol 1, fig 5) were not exposed to ischemia-reperfusion, but to a time-matched normal perfusion. After these periods, coronary arteries were precontracted with 0.1µM U-46619 administered throughout the end of the experiment. 15 min after the beginning of U-46619 infusion, the endothelial function was evaluated by the vasodilatation produced by 10µM serotonin (5-

HT), whereas coronary smooth muscle function was evaluated with 3μM sodium nitroprusside (SNP). These infusions were maintained for 10 min, which was long enough to reach a steady state. A washout period of 10 min was allowed between each infusion. Vasodilatation was evaluated by computing percent changes in coronary resistance (coronary perfusion pressure divided by coronary flow), measured immediately before each drug infusion, and after a new steady state. The concentrations of 5-HT and SNP were determined in preliminary dose-response experiments to produce near-maximal vasodilatation.

Sham, ischemic, and ischemic preconditioning hearts were treated with either 1μM SR141716A, 3μM AM630, 1μM SR144528 or their respective vehicles starting after the 20-min stabilization period, and lasting throughout the 30-min partial ischemic period (protocols 1-3, fig 5). Drug infusion was stopped upon reperfusion.

Cannabinoid perfusion protocol

In additional experimental series, the effect of perfusions of exogenous 300nM 2-AG, 300nM PEA, 100nM-1μM AEA, or their respective vehicle were compared with that of ischemic preconditioning. The animals were assigned to one of two different experimental protocols (protocols 4 and 5, fig 5). The hearts in all groups were subjected to a 20-min stabilization period. Drugs or vehicle infusion was then started, followed by an additional 15-min perfusion period. The ischemic groups (protocol 5, fig 5) were subjected to a 30 min of partial ischemia (flow rate 1 ml/min) prior to a 20-min reperfusion period. In these groups, hearts were pretreated with either 1μM SR141716A, 1μM SR144528 or vehicles starting after the 20-min stabilization period, in order to expose the hearts to the antagonist 15 min before the exogenous cannabinoid. The antagonist perfusion lasted throughout the 30-min low-flow ischemic period, and was stopped upon reperfusion. Cannabinoids were perfused for 45 min, fifteen min before the 30 min low-flow ischemia. The sham groups (protocol 4, fig 5) were not exposed to ischemia-reperfusion, but to a time-matched normal

perfusion. Endothelial and smooth muscle functions were evaluated as described above.

Statistical analysis

Values represent the mean " SEM. Statistical significance of differences between means was evaluated by a two way analysis of variance with Scheffé post-hoc test. In the presence of an interaction between the different groups, one way analyses of variance were used for each group. A commercially available software (Systat for Windows version 6.1) was used. Only probability values (p) smaller than 0.05 were considered to be statistically significant.

Drugs

SR144528 and SR141716A were a kind gift from Sanofi Recherche (Montpellier, France); PEA and AM630 were provided by Tocris Cookson (Ballwin, MO, USA). All other drugs were obtained from Sigma-Aldrich (Mississauga, Ont, Canada). AM630 (9.9mM), was prepared in 1ml 100 % dimethylsulphoxide (DMSO) and 1 ml propylene glycol. SR144528 and SR141716A (10mM) were prepared in 1ml 100 % DMSO. All these stock solutions were diluted in bidistilled water to obtain the desired final concentrations. Anandamide (1mM) was diluted in 1ml propylene glycol and 9 ml of Krebs-Henseleit buffer. 2-AG (13.2 mM) and PEA (16.7mM) were dissolved in anhydrous ethanol and diluted in Krebs-Henseleit buffer to obtain the desired final concentration. All these stock solutions were diluted in Krebs-Henseleit buffer to obtain the desired final concentrations. U-46619 (9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$, 28.5 mM) was dissolved in 100% ethanol and diluted in Krebs-Henseleit buffer to obtain the desired final concentration. Ethanol (0.003%) and DMSO (0.02%), at the concentration obtained in the final dilution, had no effect on any of the hemodynamic variables studied and on the dilator responses to 5-HT and SNP. All the other drugs were dissolved directly in Krebs-Henseleit buffer.

Results

Ischemic preconditioning groups

Coronary resistance measured just before 0.1μM U-46619 perfusion (n=25) was 5.94 " 0.29 mmHg min ml^{-1}, for a coronary flow rate of 6.70 " 0.20 ml min^{-1} g^{-1} (mean heart weight of 1.90 " 0.05 g). Infusion of U-46619 (0.1μM, n=25) induced a significant ($p<0.05$) vasoconstriction in all groups of hearts (sham, ischemia, and IPC). Perfusion of 10μM 5-HT produced a decrease in coronary resistance of -25.2 " 3.3% in sham hearts (n=8). 30 min of low-flow ischemia (n=8) significantly diminished the 5-HT-induced vasodilatation by more than half (fig 6A). One period of IPC (n=9) prevented the deleterious effect of ischemia on endothelium-dependent vasodilatation: the vasodilatation produced by 5-HT in preconditioned hearts was comparable to that observed in hearts not subjected to ischemia (fig 6A). Endothelium-independent vasodilatation to 3μM SNP was not affected by ischemia and was found to be comparable in the three groups of hearts (sham, ischemia, and IPC, fig 6B).

SR141716A (1μM), a selective antagonist of CB_1 receptors produced no significant change in coronary resistance (data not shown) Infusion of U-46619 (0.1μM, n=24) induced a significant ($p<0.05$) vasoconstriction (11.73 ± 0.51mmHg min ml^{-1}). Vasodilatation produced by 10 μM 5-HT (-24.7 ± 2.6% in sham hearts, n=10) was markedly reduced in the ischemic group (n=8) (fig 6C). IPC in SR141716A-treated hearts (n=6) was unable to prevent the deleterious effect of ischemia on 5-HT-induced vasodilatation (fig 6C). Vasodilatation to 3μM SNP was comparable in the three SR141716A-treated groups (sham, ischemic, and IPC, fig 6D).

AM630 (3μM), another selective antagonist of CB_1 receptors produced no significant change in coronary resistance (data not shown). Infusion of U-46619 (0.1μM, n=22) induced a significant ($p<0.05$) vasoconstriction (11.57 ± 0.52 mmHg min ml^{-1}). Vasodilatation produced by 10 μM 5-HT (-25.2± 3.3% in sham hearts, n=8) was

severely reduced in the ischemic group (n=8) (fig 6E). IPC in AM630-treated hearts (n=6) was unable to prevent the deleterious effect of ischemia on 5-HT-induced vasodilatation (fig 6E). Vasodilatation to 3μM SNP was comparable in the three AM630-treated groups (sham, ischemic, and IPC, fig 6F).

SR144528 (1μM), a selective antagonist of CB_2 receptors produced no significant change in coronary resistance (data not shown). Infusion of U-46619 (0.1μM, n=24) induced a significant ($p<0.05$) vasoconstriction (12.09± 0.63 mmHg min ml^{-1}). Vasodilatation produced by 10 μM 5-HT (-25.0± 2.9% in sham hearts, n=9) was markedly depressed in the ischemic group (n=8) (fig 7C). IPC in SR144528-treated hearts (n=6) was unable to prevent the deleterious effect of ischemia on 5-HT-induced vasodilatation (fig 7C). Vasodilatation to 3μM SNP was comparable in the three SR144528-treated groups (sham, ischemic, and IPC, fig 7D).

Exogenous prostaglandin groups

Perfusion with AEA (100nM-1μM) produced no significant effect in coronary resistance when measured five min after the beginning of its perfusion (data not shown). Infusion of U-46619 (0.1μM, n=18) induced a significant ($p<0.05$) vasoconstriction in AEA-treated hearts (13.37 ± 1.14 mmHg min ml^{-1}). 30 min of low-flow ischemia significantly diminished the 5-HT-induced vasodilatation by more than half in untreated hearts (fig 8A). Treatment with AEA (100nM-1μM), starting 15 min before ischemia and lasting 45 min, was unable to preserve the vasodilatation produced by 10μM 5-HT in ischemic hearts (fig 8A). Vasodilatation to 3μM SNP was comparable in all AEA-treated hearts (fig 8B).

Perfusion with PEA (300nM) produced no significant effect in coronary resistance when measured five min after the beginning of its perfusion (data not shown). Infusion of U-46619 (0.1μM, n=17) induced a significant ($p<0.05$) vasoconstriction in PEA-treated hearts (12.70 ± 1.27 mmHg min ml^{-1}). 30 min of low-flow

ischemia significantly diminished the 5-HT-induced vasodilatation by more than half in untreated hearts (fig 8C). Treatment with PEA (300nM), starting 15 min before ischemia and lasting 45 min, preserved the vasodilatation produced by 10μM 5-HT in ischemic hearts (fig 8C). Vasodilatation to 3μM SNP was comparable in all PEA-treated hearts (fig 8D). Treatment with PEA in SR141716A or in SR144528-pretreated hearts was unable to prevent the deleterious effect of ischemia on 5-HT-induced vasodilatation (fig 8C).

Perfusion with 2-AG (300nM) produced no significant effect in coronary resistance when measured five min after the beginning of its perfusion (data not shown). Infusion of U-46619 (0.1μM, n=18) induced a significant ($p<0.05$) vasoconstriction in 2-AG-treated hearts (13.65 ± 1.47 mmHg min ml^{-1}). 30 min of low-flow ischemia significantly halved the 5-HT-induced vasodilatation by more than half in untreated hearts (fig 8E). Treatment with 2-AG (300nM), starting 15 min before ischemia and lasting 45 min, preserved the vasodilatation produced by 10μM 5-HT in ischemic hearts (fig 8E). Vasodilatation to 3μM SNP was comparable in all 2-AG-treated hearts (fig 8F). Treatment with 2-AG perfusion in SR141716A-pretreated hearts still prevented the deleterious effect of ischemia on 5-HT-induced vasodilatation (fig 8E). In contrast, treatment with 2-AG in SR144528-pretreated hearts was unable to prevent the deleterious effect of ischemia and reperfusion on 5-HT vasodilatation (fig 8E).

Discussion

In the present study, we have assessed the contribution of cannabinoids in the protective effect of IPC on endothelial function in the rat heart. The major findings are 1) that IPC with a single short period of ischemia prevents endothelial dysfunction produced by ischemia-reperfusion in rat hearts partially via the release of cannabinoids 2) this protection can be blocked by a pre-treatment with SR141716A, AM630 or SR144528, and 3) 45-min perfusion with 2-AG, PEA, but not AEA, starting 15 min before ischemia mimic the

beneficial effect of IPC on endothelial function in rat coronary arteries. This protective effect was blocked by SR144528 and SR141716A in the case of PEA, and by SR144528 only with 2-AG perfusion.

Effect of ischemic preconditioning on endothelial dysfunction

In the present study, the ischemic conditions (flow rate and duration) were selected in order to observe a selective endothelial dysfunction. This was confirmed by the fact that the endothelium-dependent and NO-mediated [28] vasodilatation of coronary arteries to 5-HT was drastically decreased after ischemia-reperfusion insult, whereas the same vessels retained the ability to dilate to SNP, an endothelium-independent vasodilator. We have reported earlier [9] that IPC could prevent the reduction in the vasodilatation to 5-HT after ischemia-reperfusion, suggesting that ischemic preconditioning could protect endothelial function in coronary arteries. Such a protection was observed in the present study.

Role of cannabinoids in ischemic preconditioning

Cannabinoids exert several physiological and pharmacological actions, including suppression of the immune system [8; 12], antioxydative effects [13], and cardiovascular actions [14; 15]. However, to our best knowledge, little is known about the role played by endogenous cannabinoids in the protective effect of IPC. Therefore, we tested, using SR141716A and AM630 (selective CB_1 receptor antagonists), and SR144528 (selective CB_2 receptor antagonist), whether endogenous cannabinoids were involved in the protection afforded by IPC to the endothelium. In SR141716A, AM630, and SR144528-treated and preconditioned groups, the vasodilatation to 5-HT was almost totally abolished whereas the vasodilatation to SNP was not significantly different from that observed in the sham groups. These data suggest that endogenously produced cannabinoids play a role in the endothelial protection afforded by IPC by acting on CB_1 or CB_2 receptors. This is the first time that endogenous cannabinoids have been reported to be mediators of IPC.

Protective effect of exogenous cannabinoids

To confirm the contribution of cannabinoids in the endothelial protection afforded by IPC, the effect of exogenous perfusion with a low concentration of 2-AG, PEA, and AEA, on the endothelial function following ischemia-reperfusion was studied. 2-AG and PEA, but not AEA, perfused, 15 min before and throughout ischemia prevented the ischemia-induced reduction in the vasodilatation to 5-HT. Only the CB_2 receptor antagonist abolished the protective effect of 2-AG, whereas both the CB_1 and CB_2 receptor antagonists abolished that of PEA. Thus, taken together these data suggest that 2-AG and PEA, can mimic the protective effect of IPC on the endothelial function via the activation of CB_2 receptors for 2-AG and PEA, and via CB_1 receptors for PEA. The lack of effect of AEA may be explained by the short life span of this compound, which undergoes rapid inactivation in tissues [18; 19].

In the brain, delta 9-tetrahydrocannabinol, cannabinol, cannabidiol, and AEA increased the activity of protein kinase C (PKC) [39; 40]. Randall et al reported that AEA produced vasorelaxation in isolated rat hearts by an hyperpolarisation, this being blocked by non-selective potassium channel blockers [41]. Also, some groups demonstrated that endogenous cannabinoids produced a concentration-related increase in the activity of the mitogen-activated protein (MAP) kinases [42; 43]. PKC [35; 36], ATP sensitive potassium (K_{ATP}) channels [28], MAP kinases [44] activation have been implicated in the mechanisms of the protection afforded to the myocardium by IPC. Activated PKC and MAP kinases may phosphorylate secondary effectors, which would be responsible for the protective effects of cannaninoids. In the myocardium, activation of K_{ATP} channels has been linked to cardioprotection, possibly through a reduction in intracellular Ca^{2+} levels [34], prevention of mitochondrial Ca^{2+} overload or preservation of the myocardial energy status [45]. In endothelial cells, activation of K_{ATP} channels produces hyperpolarisation, which increases the

electrochemical gradient for Ca^{2+} entry [46], resulting in a enhanced nitric oxide release [47]. Currently, little is known about the pathways stimulated by the activation of CB_1 and CB_2 receptors in the heart. Thus, for the moment, mechanisms proposed in this paper are only speculative, further experiments are needed to assess the pathways implicated during the activation of cannabinoid receptors.

In conclusion, these data suggest that IPC affords protection to the endothelial function against subsequent ischemic injury in the intact coronary circulation of the rat. The reduced protective effect of IPC in presence of SR141716A, AM630, and SR144528 during IPC suggest, for the first time, that this protection may be mediated partially via the production of endogenous cannabinoids. Exogenous perfusion of 2-AG and PEA can afford protection to the endothelial function against the deleterious effect of ischemia-reperfusion via an activation of their specific receptors.

It is believed that a cannabinoid-like compound achieving an effect equivalent to the effect of 30nM to 1mM of PEA or 2-AG, preferably 100nM-500nM of the same are contemplated.

EXAMPLE 3

CB_1, and CB_2 receptor agonists as cardioprotective compounds

Based on the data obtained with isolated rat hearts, we contemplate the use of cannabinoid mimetic agents, like receptor agonists including compounds like L768242, L759633, L759656, JWH-015, JWH-051 and JWH-057, or inhibitors of the uptake or degradation of endocannabinoids, would be strong cardioprotective or anti-ischemic agents. These agents have a potential for the treatment or the prevention of ischemic diseases.

The above receptor agonists are tested in anaesthetized pigs and dogs to determine which are the best cardioprotective candidate. The activity of the compounds useful as inhibitors of endocannabinoid uptake or

degradation is evaluated for their capacity to reduce infarct size.. Non-selective or selective CB_1 and CB_2-receptor agonists are tested.

Methodology

Animal preparation

Adult mongrel dogs of either sex weighing between 20 and 25 kg are housed at a constant temperature of 20-22 °C on a 12 h light/dark cycle and provided with food and water *ad libitum*. They are anaesthetized with sodium thiopental (25 mg/kg i.v.) and alpha-chloralose (80 mg/kg i.v. followed by 15-20 mg/ kg i.v. hourly), and ventilated artificially with room air through an endotracheal tube by means of a Harvard pump (model 607). Atelectasis is prevented by maintaining an end-expiratory pressure of 5 to 7 cm H_2O with a trap. Arterial blood pH, Pco_2 and Po_2 are monitored at selected intervals by a blood-gas analyzer (Copenhogen, ABC1) and maintained within normal physiological limits (pH 7.35 to 7.45; Pco_2, 30 to 35 mm Hg and Po_2, 85 to 100 mm Hg) by adjustment of the respiration rate or by i.v. administration of 1.5% sodium bicarbonate, if necessary. Body temperature is maintained at 38 ± 1□C with a heating pad. One femoral vein and one femoral artery are cannulated for drug injection and arterial blood pressure recording, respectively. A Millar Mikrotip catheter are placed inside the left ventricle through a carotid artery for left ventricular pressure recording. A left thoracotomy is performed at the fifth intercostal space, the lung carefully retracted, the pericardium incised, and the heart suspended in a cradle. A proximal portion of the left anterior descending coronary artery (LAD) distal to the first diagonal branch is isolated from surrounding tissue, and an ultrasonic perivascular probe (Transonic Systems Inc.) is placed around the vessel. An occluding snare (umbilical tape) is placed distal to the flow probe ensuring that no branches are between the flow probe and the occluding snare. The left atrium is cannulated through the appendage for radioactive microsphere

injection. Finally, a radio-opaque catheter used for blood sampling is passed, under fluoroscopic monitoring, through the left jugular vein until the tip of the catheter is 1-2 cm deep inside the coronary sinus. Localization of this catheter is confirmed by bolus injections of a radio-opaque contrasting agent.

Basal heart rate in this preparation is normally around 130 bpm (48). If heart rate fall below 120 bpm (either spontaneously or due to some of the agents studied), the heart is paced at 130 bpm with rectangular pulses of 4 msec duration and with a voltage twice the threshold through bipolar electrodes clipped to the left atrial appendage.

The electrocardiogram (lead DII), heart rate, mean arterial pressure, left ventricular pressure, and its first derivative (dP/dt), are recorded on a Nihon Kohden RM-6000 polygraph. Coronary blood flow is measured with a Transonic T208 flowmeter, with the mean signal recorded on the polygraph. All signals are simultaneously recorded on a Biopac Systems model M100SWS data acquisition system.

The preparation of pigs are as close as possible from that of dogs, beside some differences imposed by the particularities of this species. Young Landrace pigs are anaesthesed starting with an i.m. injection of ketamine 20 mg/kg and xylazine 2 mg/kg. Once the animal has "tranquilized", anaesthesia will continue with pentobarbital (30 mg/kg i.v.) and alpha-chloralose (60 mg/kg i.v. followed by 15-20 mg/kg i.v. hourly). Due to anatomical particularities, a mid-line sternotomy, instead of a thoracotomy, gives access to the heart. The rest of the procedure is similar or identical.

Experimental protocols

After all surgical procedures, a stabilisation period of 30 min is allowed. All hemodynamic parameters are measured, and aortic and coronary sinus blood samples taken, for determination of the baseline values. Drugs (or vehicles) are administered and a second 15 min stabilisation period allowed before a second set of aortic and coronary sinus blood samples taken. The LAD is occluded for either 20, 40, or 80 min, and reperfusion allowed for 3 hours. Animals victims of VF are defibrillated with low-energy d.c. pulses applied directly to the heart.

At the end of the 3-hour reperfusion period, the LAD are cannulated. To determine the anatomic area at risk (AR) and the non ischemic area, 5 ml of Patent blue dye and 5 ml of saline are injected at equal pressure into the left atrium and LAD, respectively. The heart is immediately fibrillated and removed. The left ventricle is dissected and sliced into serial transverse sections 6- to 7-mm wide. The unstained ischemic area and the blue-stained normal area are separated, and both regions are incubated at 37□C for 15 min in 1% 2,3,5-triphenyl tetrazolium chloride (TTC) in 0.1 M phosphate buffer adjusted to pH 7.4. The TTC stains the non infarcted myocardium brick red, indicating the presence of a formazin precipitate that results from the reduction of TTC by dehydrogenase enzymes in viable tissue. After storage overnight in 10% formaldehyde, infarcted and non infarcted tissues within the AR are separated and determined gravimetrically. Infarct size is expressed as a percent of the AR.

Regional myocardial blood flow is measured by the radioactive microsphere technique (49)(50). Microspheres are administered halfway during the occlusion period and at the end of reperfusion. Carbonized plastic microspheres (15 micrometers diameter) labelled with ^{141}Ce or ^{95}Nb is suspended in isotonic saline with 0.01% Tween 80 added to prevent aggregation. The microspheres are ultrasonicated for 5 min and vortexed for another 5 min before injection. One ml of the

microsphere suspension (2 to 4 □ 10^6 spheres) are given through the left atrial catheter and flushed by 5 ml of saline. A reference blood flow sample is drawn from the right femoral artery at a constant rate of 9.4 ml/min starting 30 sec before microsphere injection and continuing for 3 min. The next day, the tissue slices are sectioned into sub-epicardium, mid-myocardium and sub-endocardium of non ischemic (3 pieces) and ischemic (5 pieces) regions. Transmural pieces are obtained from the center of several transverse sections used to determine the AR and they are at least 1 cm from the perfusion boundaries as indicated by Patent blue dye. All samples are counted in a gamma-counter to determine the activity of each isotope in each sample. The activity of each isotope is also determined in the reference blood flow samples. Myocardial blood flow is calculated with the equation $Q_m = Q_r x C_m / C_r$, where Q_m is myocardial blood flow (in ml/min per gram of tissue), Q_r is the rate of withdrawal of the reference blood flow (9.4 ml/min in this protocol), C_r is the activity of the blood flow sample (cpm) and C_m is the activity of the tissue sample (cpm/g). Transmural blood flow is calculated as the weighted average of the three layers in each region.

Drugs tested

To test whether cannabinoid mimetic agents can protect the heart against ischemia, the effect of AM404, an inhibitor of anandamide transport is evaluated (52)(53), on infarct size in anaesthetized dogs and pigs subjected to 20, 40, and 80 min of ischemia. Since the degradation of anandamide by FAAH is mainly intracellular, inhibition of the anandamide transporter by AM404 is likely to increase the concentration of anandamide, and possibly 2-arachidonylglycerol (52), resulting in a reduced infarct size in groups with longer ischemic periods. Cannabinoid receptor agonists are also tested. In isolated rat hearts, the cardioprotective effect of palmitoylethanolamine and

2-arachidonylglycerol is reversed by th CB_2 receptor antagonist. Therefore, antagonists like L768242, L759633, L759656, JWH-015, JWH-051 and JWH-057, alone or in combination are tested for their potency and selectivity for CB, and CB_2 receptors. These analogues have a higher affinity for CB_2 than CB_1 receptors, are metabolically more stable and have a longer duration of action *in vivo*. These compounds should reduce infarct size, additional experiments and confirmation will be performed with them in the presence of a pre-treatment with either SR141761A or SR144528 antagonists.

For purpose of clarity the following chemical definitions correspond to the above-cited compounds:

SR141716A
[N-(piperidin-l-yl)-5-(4-chlorophenyl)-1-(2,4-di-chlorophenyl)-4-methyl lH-pyrazole-3-carboxamide hydrochloride]

SR144528
{N-[(1S)-endo-1,3,3-trimethyl bicyclo[2.2.l] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-l-(4-methyl-benzyl)-pyrazole-3-carboxamide}

L759633
[(6aR, l0aR)-3-(1,1-dimethyl-heptyl)-1-methoxy-6,6,9-trimethyl-6a, 7 10, 10a-tetrahydro-6H-benzo[c]chromene]

L759656
[(6aR, 10aR)-3-(1,1-dimethyl-heptyl)-1-methoxy-6,6-dimethyl-9-methylene-6a, 7, 8, 9, 10, 10a-hexahydro-6H-benzo[c]chromene]

L768242
1-(2, 3-dichlorobenzoyl)-2-methyl-3-(2-[1-morpholino]ethyl)-5-methoxyindole

JWH-015
2-methyl-1-propyl-3-(1-naphthoyl)indole

JWH-051
1-deoxy-11-hydroxy-delta8-THC-DMH

JWH-057
1-deoxy-delta8-THC-DMH

Data analysis and statistics

Statistical significance of differences between means of all parametric data (ratio infarct size/AR, hemodynamic parameters, etc.) are evaluated with an analysis of variance (Systat® 9 for Windows®). The incidence of VT and VF between groups are compared with a two-way crosstabulation followed by a Pearson Chi^2 test (or the Fisher exact test if the conditions to apply the former are not met). Beside the comparison of the infarct size/AR ratio, correlations between the infarct size/AR ratio and the transmural collateral blood flow (determined with the microspheres) is calculated for each group and compared with an analysis of covariance. A significant downward shift of the regression line confirms that the reduction in infarct size is independent of changes in collateral blood flow.

This invention has been described hereinabove and modifications are readily apparent to the skilled artisan and can be practice without departing from the spirit and the teachings of the invention. These modifications are within the scope of this invention as defined in the appended claims.

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WHAT IS CLAIMED IS:

1. A method for protecting endothelial cells or cardiomyocytes against damages caused by ischemia, which comprises the step of contacting the cells or myocytes with a protective amount of a compound capable of increasing the extracellular concentration of cannabinoids or of mimicking the action of endogenous cannabinoids.

2. The method of claim 1, wherein said compound is capable of increasing the synthesis of endogenous cannabinoids or of decreasing the degradation thereof, or of decreasing the uptake of cannabinoids, or is a cannabinoid active metabolite, a cannabinoid analog or a cannabinoid agonist.

3. The method of claim 1, wherein said compound is a cannabinoid administered exogenously.

4. The method of claim 1 or 3, wherein said cannabinoid is either palmitoyl ethanolamide or 2-arachidonyl glycerol, or a mixture of both.

5. The method of any one of claims 1 to 3, wherein said cannabinoid is either JWH-015, JWH-051, JWH-057, L 768242, L 759633 or L 759656, or any mixture thereof.

6. The use of a cannabinoid-like compound in the making of a medication for protecting endothelial cells and cardiocytes against damage caused by ischemia.

7. The use of claim 6, wherein said compound is capable of increasing the synthesis of endogenous cannabinoids or of decreasing the degradation thereof, or of decreasing the uptake of cannabinoids, or is a cannabinoid active metabolite, a cannabinoid analog or a cannabinoid agonist.

8. The use of claim 6 or 7, wherein said cannabinoid is either palmitoyl ethanolamide or 2-arachidonyl glycerol, or a mixture of both.

9. The use of claim 6 or 7, wherein said cannabinoid is either JWH-015, JWH-051, JWH-057, L 768242, L 759633 or L 759656, or any mixture thereof.

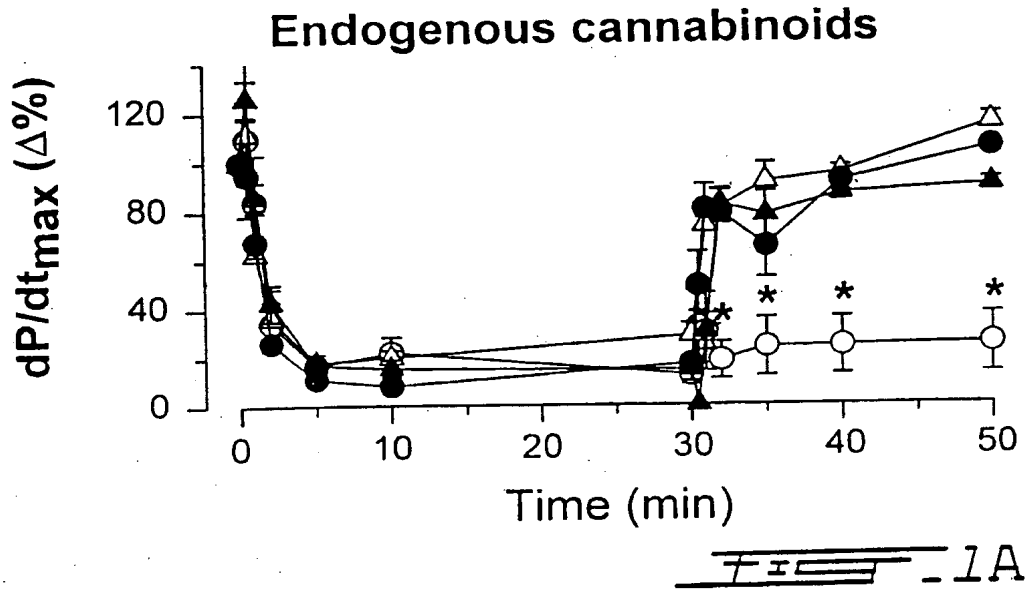
Endogenous cannabinoids
dP/dtmax (Δ%)
120
80
40
0
0
10
20
30
40
50
Time (min)

Fig. 1A


ISCHEMIA 1 ML/MIN
REPERFUSION

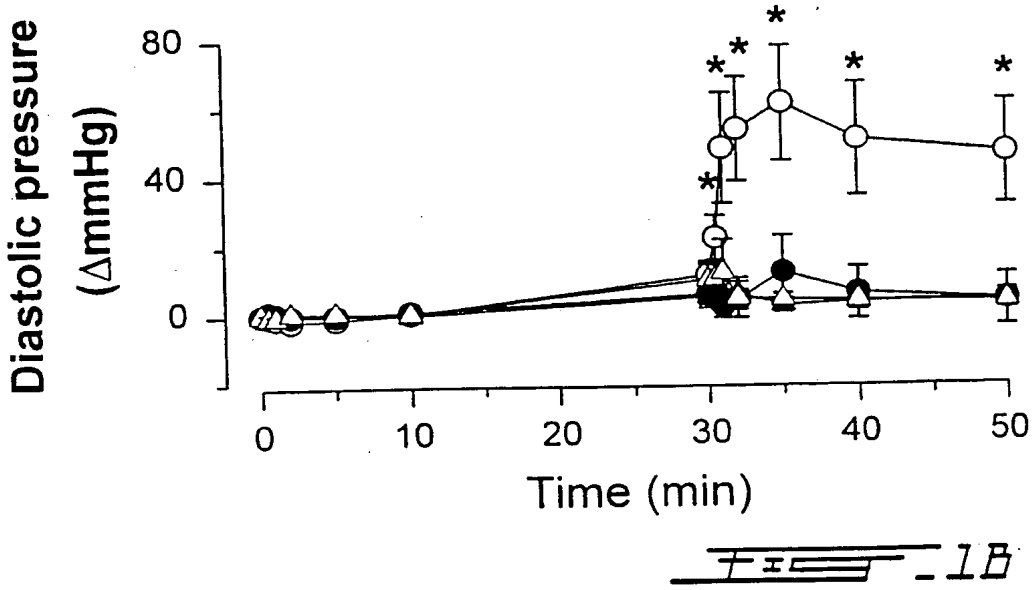
Diastolic pressure
(ΔmmHg)
80
40
0
0
10
20
30
40
50
Time (min)

Fig. 1B

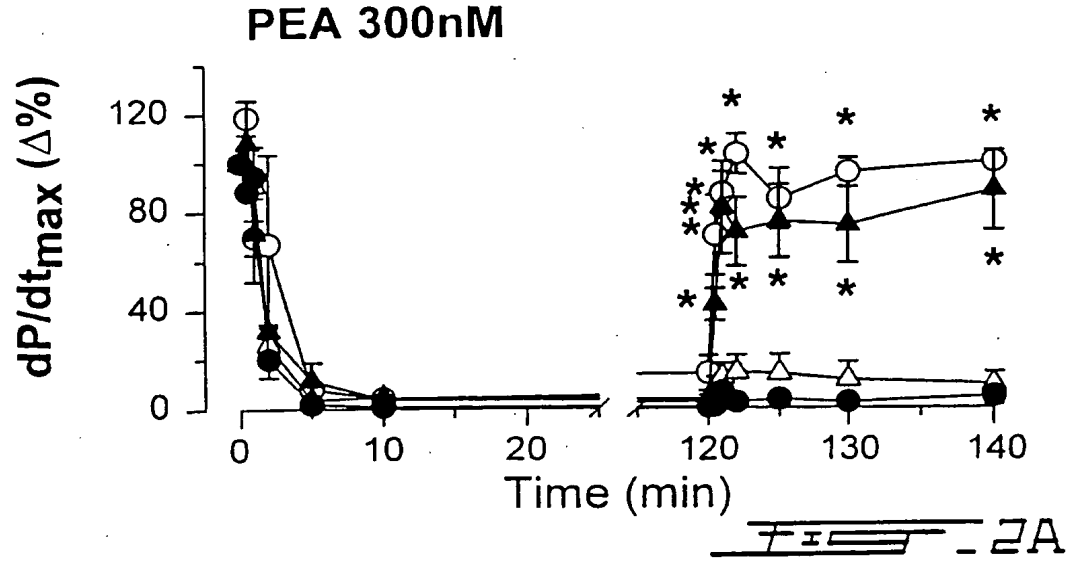

FIG - 2A

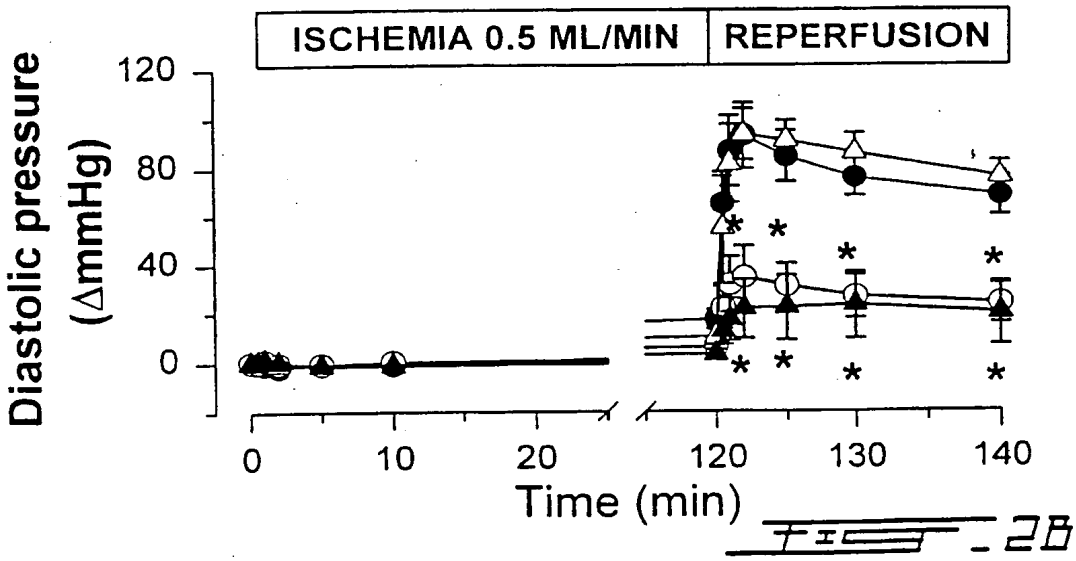

FIG - 2B

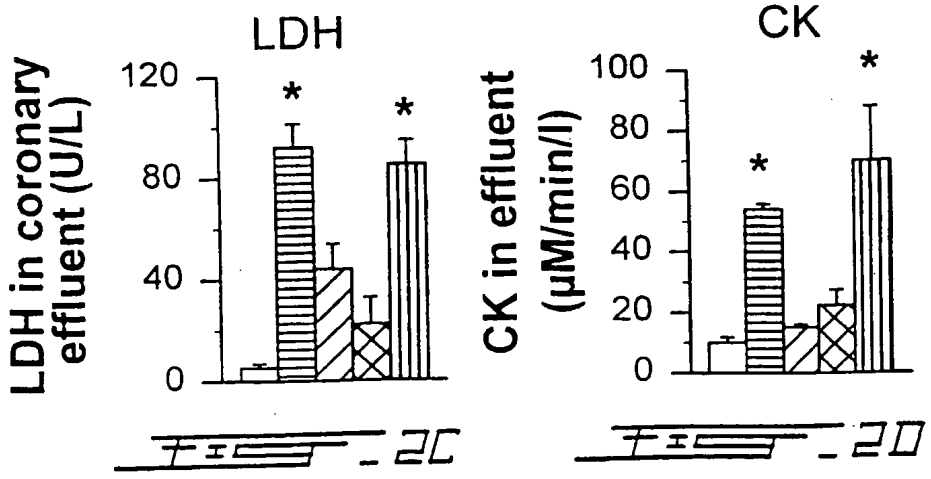

FIG - 2C FIG - 2D

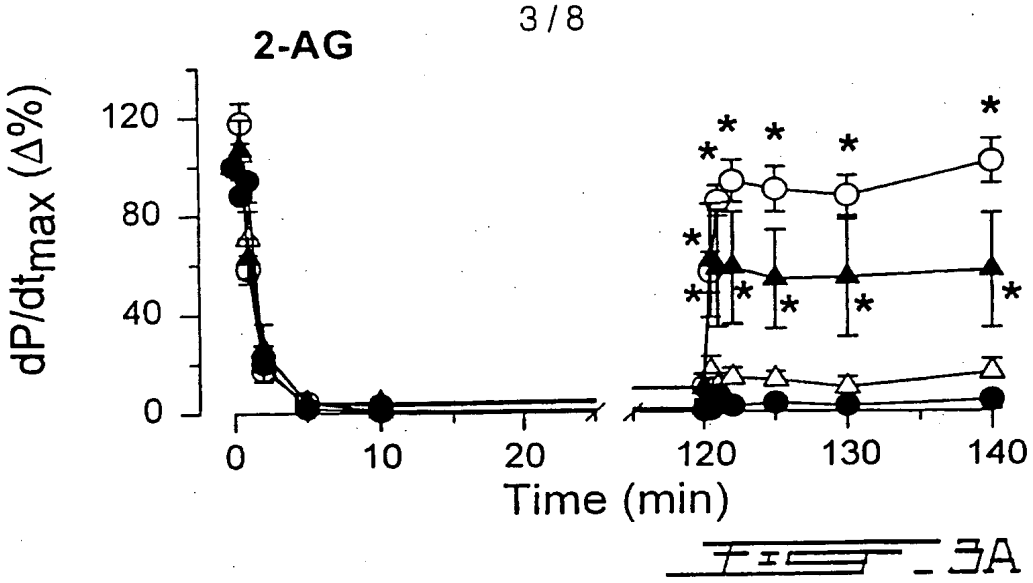

Fig 3A

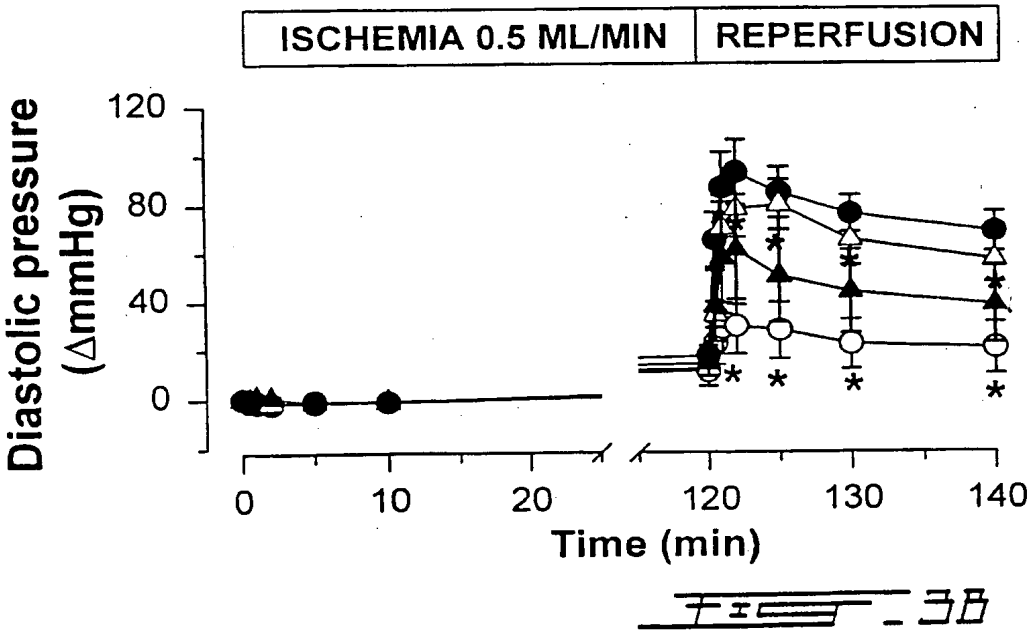

Fig 3B

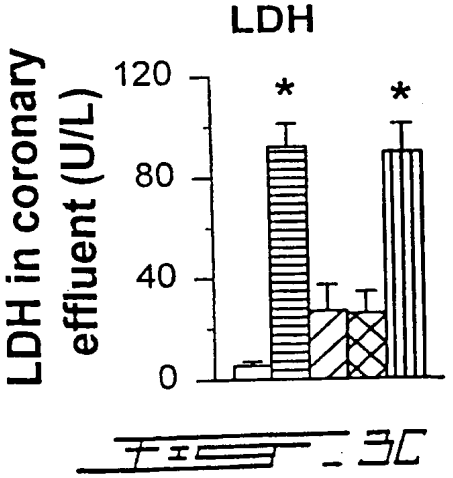

Fig 3C

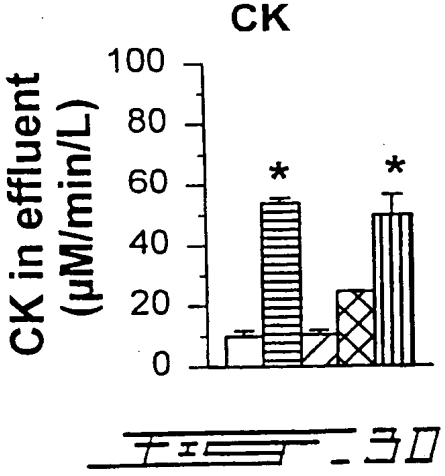

Fig 3D

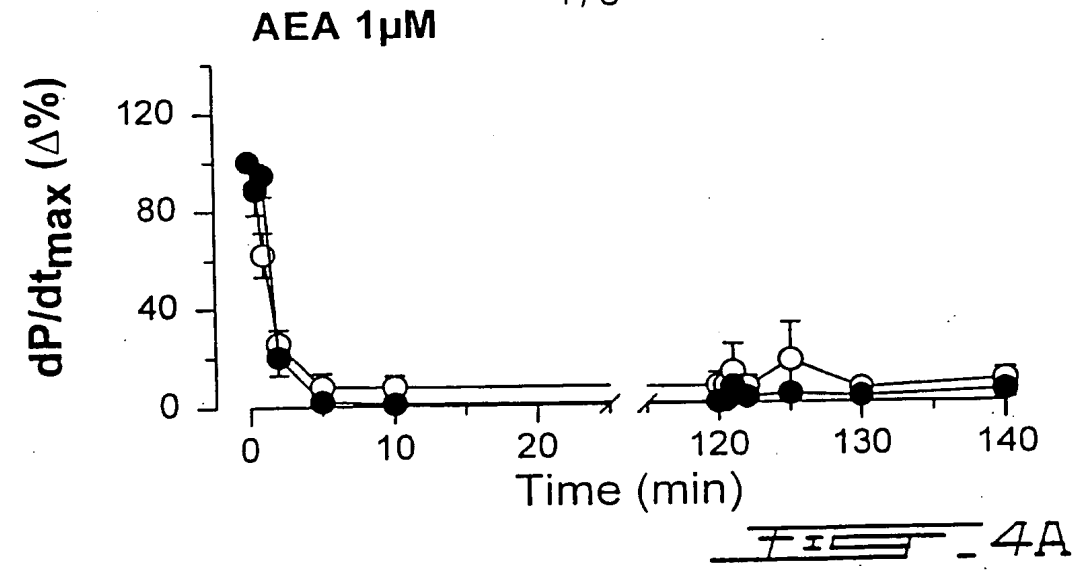

Fig. 4A

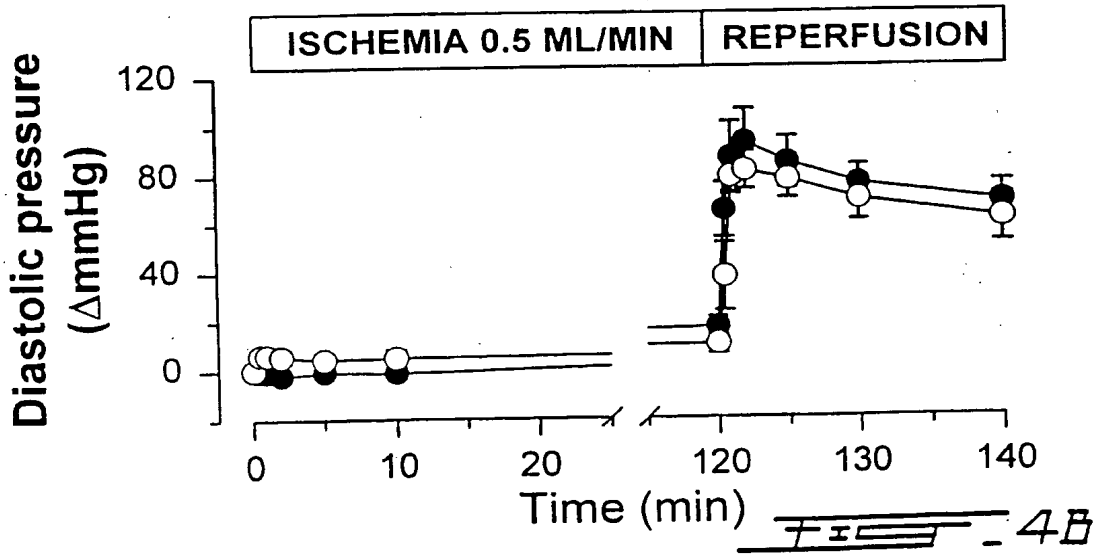

Fig. 4B

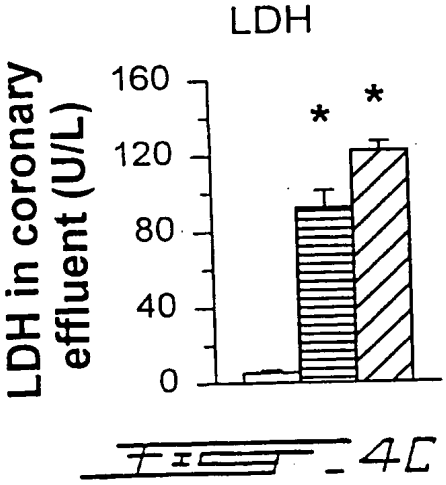

Fig. 4C

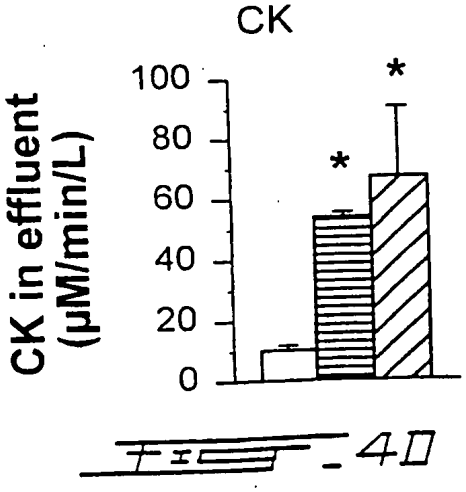

Fig. 4D

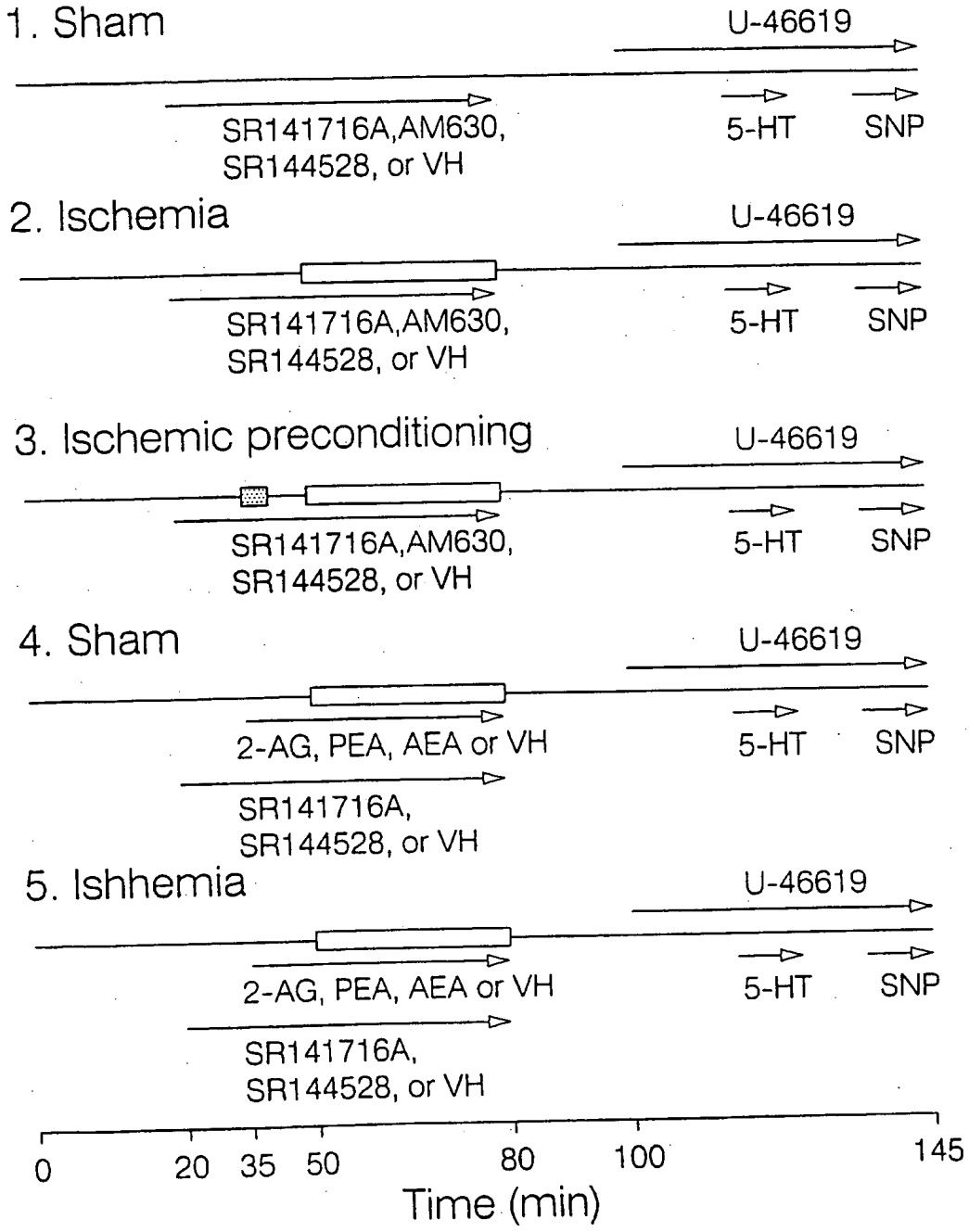
1. Sham
U-46619
SR141716A,AM630,
SR144528, or VH
5-HT
SNP
2. Ischemia
U-46619
SR141716A,AM630,
SR144528, or VH
5-HT
SNP
3. Ischemic preconditioning
U-46619
SR141716A,AM630,
SR144528, or VH
5-HT
SNP
4. Sham
U-46619
2-AG, PEA, AEA or VH
5-HT
SNP
SR141716A,
SR144528, or VH
5. Ishhemia
U-46619
2-AG, PEA, AEA or VH
5-HT
SNP
SR141716A,
SR144528, or VH
0
20
35
50
80
100
145
Time (min)
Complete ischemia (0 ml min-1)
Low-flow ischemia (1 ml min-1)
Drug perfusion

FIG 5

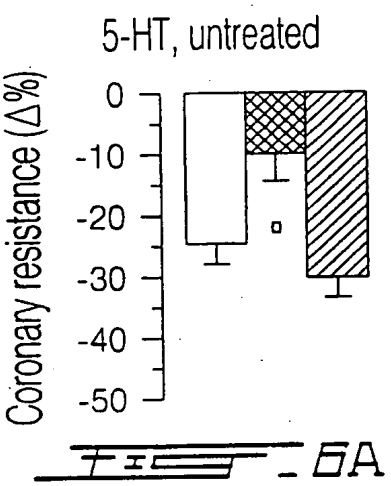

FIG 6A

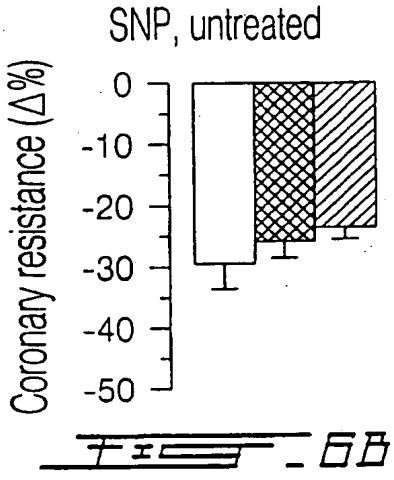

FIG 6B

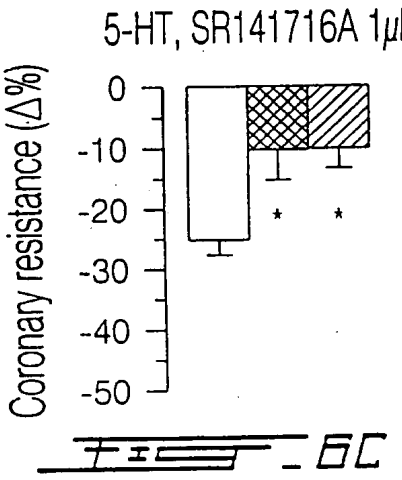

FIG 6C

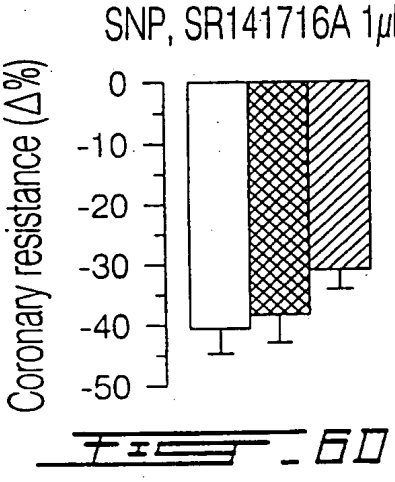

FIG 6D

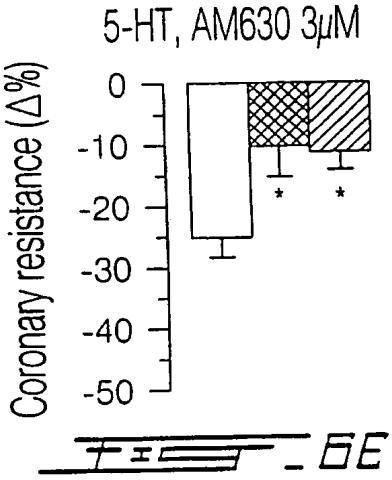

FIG 6E

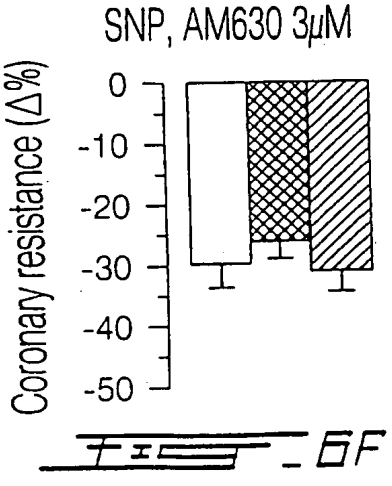

FIG 6F

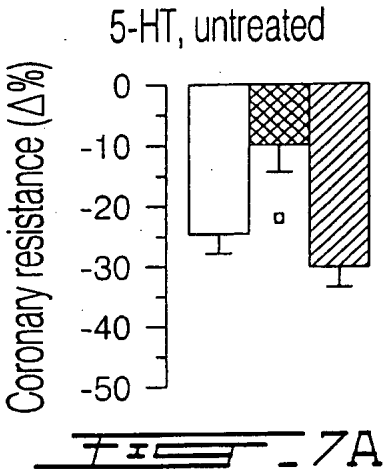

Fig. 7A

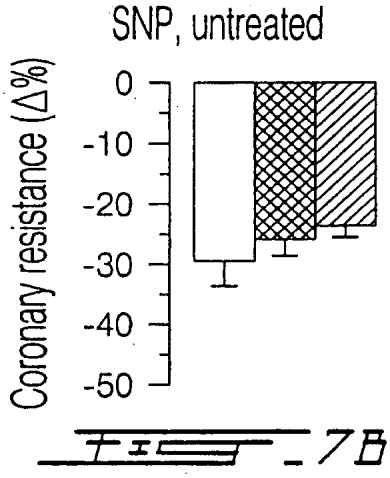

Fig. 7B

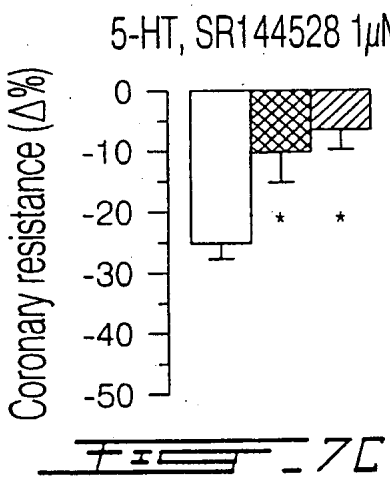

Fig. 7C

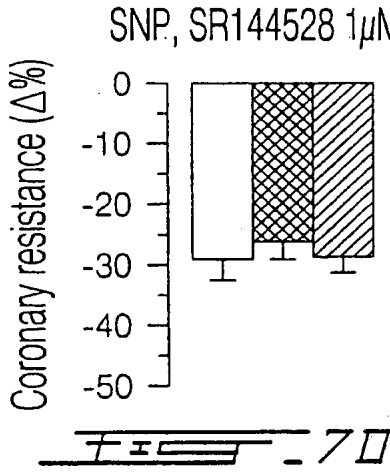

Fig. 7D

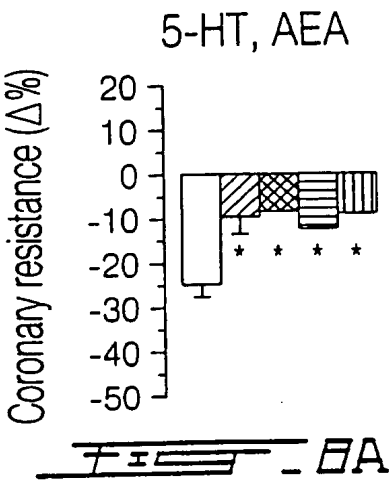

FIG 8A

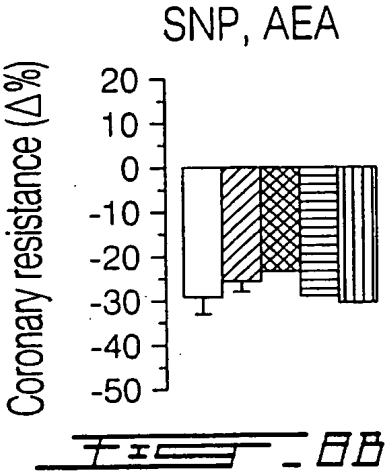

FIG 8B

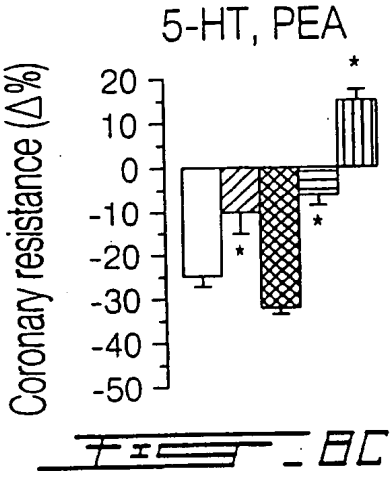

FIG 8C

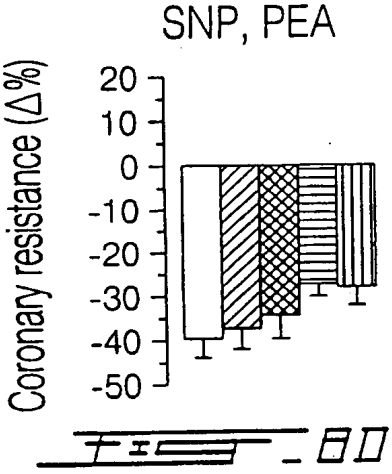

FIG 8D

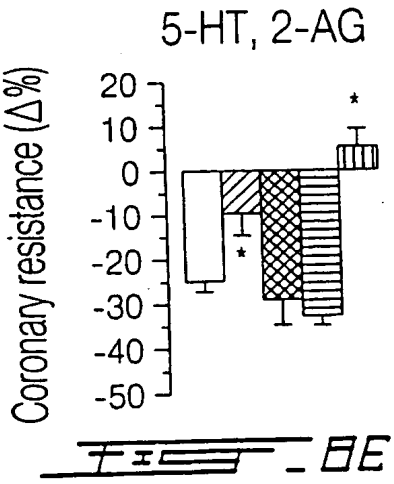

FIG 8E

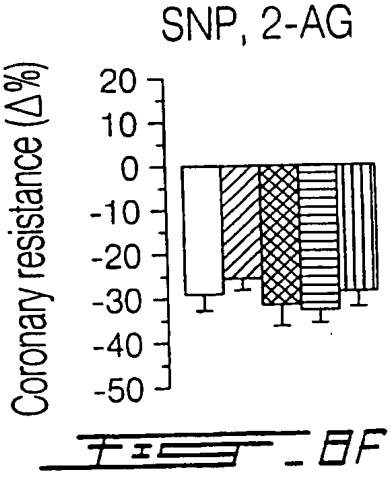

FIG 8F

INTERNATIONAL SEARCH REPORT

Internati. Application No
PCT/CA 00/01242

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K45/06 A61P9/10 A61K31/232 A61K31/23

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, EMBASE, WPI Data, MEDLINE, CHEM ABS Data, BIOSIS, PASCAL, SCISEARCH AIDSLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NAGAYAMA T ET AL: "CANNABINOIDS AND NEUROPROTECTION IN GLOBAL AND FOCAL CEREBRAL ISCHEMIA AND IN NEURONAL CULTURES" JOURNAL OF NEUROSCIENCE,US,NEW YORK, NY, vol. 19, no. 8, April 1999 (1999-04), pages 2987-2995, XP000920687 ISSN: 0270-6474 abstract --- -/--	4,5,8,9

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 March 2001	26.03.01
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Uiber, P

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Internatio. Application No
PCT/CA 00/01242

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	WAGNER J.A. ET AL: "Cardiovascular actions of cannabinoids and their generation during shock" JOURNAL OF MOLECULAR MEDICINE (J. MOL. MED.), 76/12 (824-836), XP000983836 Germany * p.826, Fig.1; p.833, Research propects and clinical implications *	4,5,8,9
Y	WO 99 26612 A (CHRISTENSEN SIEGFRIED B ;SMITHKLINE BEECHAM CORP (US); BENDER PAUL) 3 June 1999 (1999-06-03) * p.1, l.28-p.2, l.15; p.3, l26-27; p.4, l.5-7 *	4,5,8,9
A	US 5 624 941 A (BARTH FRANCIS ET AL) 29 April 1997 (1997-04-29) column 28, line 22 - line 26	4,5,8,9

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No. PCT/CA 00 01242

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 4,5,8,9 (fully)

Present claims 1-3, 6 and 7 are directed to a method whre the active agents are defined by reference to a desirable characteristic or property, namely a compound capable of increasing the extracellular concentration of cannabinoids or of mimicking the action of endogenous cannabinoids. Moreover, the definition for "a compound capable of increasing the extracellular concentration of cannabinoids" given in claim 2 covers various groups of compounds which act on different (aspects) of the cannabinoid metabolism/pathway but are also defined by the result to be achieved. These claims cover all methods with the compounds having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such compounds. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the compounds by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been ONLY carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the compounds explicitely defined in claims 4, 5, 8 and 9.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA 00/01242

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 4,5,8,9 (fully)
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internatio. Application No
PCT/CA 00/01242

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